JOURNAL OF BACTERIOLOGY, Mar. 2011, p. 1369–1376 Vol. 193, No. 6
0021-9193/11/$12.00 doi:10.1128/JB.00288-10

The *tib* Adherence Locus of Enterotoxigenic *Escherichia coli* Is Regulated by Cyclic AMP Receptor Protein[▽]

Shirley M. Espert,[1] Eric A. Elsinghorst,[2] and George P. Munson[1]*

Department of Microbiology and Immunology, University of Miami Miller School of Medicine, Miami, Florida,[1] and Department of Clinical Laboratory Sciences, University of Kansas Medical Center, Kansas City, Kansas[2]

Received 15 March 2010/Accepted 18 December 2010

Enterotoxigenic *Escherichia coli* (ETEC) is a Gram-negative enteric pathogen that causes profuse watery diarrhea through the elaboration of heat-labile and/or heat-stable toxins. Virulence is also dependent upon the expression of adhesive pili and afimbrial adhesins that allow the pathogen to adhere to the intestinal epithelium or mucosa. Both types of enterotoxins are regulated at the level of transcription by cyclic AMP (cAMP) receptor protein (CRP). To further our understanding of virulence gene regulation, an *in silico* approach was used to identify putative CRP binding sites in the genome of H10407 (O78:H11), an ETEC strain that was originally isolated from the stool of a Bangledeshi patient with cholera-like symptoms circa 1971. One of the predicted binding sites was located within an intergenic region upstream of *tibDBCA*. TibA is an autotransporter and afimbrial adhesin that is glycosylated by TibC. Expression of the TibA glycoprotein was abolished in an H10407 *crp* mutant and restored when *crp* was provided in *trans*. TibA-dependent aggregation was also abolished in a *cyaA*::*kan* strain and restored by addition of exogenous cAMP to the growth medium. DNase I footprinting confirmed that the predicted site upstream of *tibDBCA* is bound by CRP. Point mutations within the CRP binding site were found to abolish or significantly impair CRP-dependent activation of the *tibDB* promoter. Thus, these studies demonstrate that CRP positively regulates the expression of the glycosylated afimbrial adhesin TibA through occupancy of a binding site within *tibDBp*.

More than 100 genes in *Escherichia coli* and many other bacterial species are regulated by cyclic AMP (cAMP) receptor protein (CRP) through direct and indirect pathways (12, 33, 36, 42, 75, 76). For CRP, DNA binding is cAMP dependent and the position of a CRP binding site relative to RNA polymerase largely determines whether it functions as an activator or a repressor of a particular gene or operon. With respect to the transcription start site, CRP binding sites centered at or near −62, −72, −83, or −93 are typical for class I CRP-dependent promoters. Class II promoters have a binding site centered at or near −41.5 (16, 24, 50). Class III promoters are more complex in that they usually require an additional transcription factor or multiple CRP binding sites (24). In comparison to class I and class II promoters, class III and CRP-repressed promoters display a greater range of binding site positions (33).

In addition to regulating the expression of metabolic and housekeeping genes, CRP has been shown to regulate the expression of many virulence factors. For example, CRP positively regulates the expression of Pla, a surface-exposed protease of *Yersinia pestis* that is necessary for the establishment of primary pneumonic plague and facilitates dissemination of the bacterium during bubonic plague (46, 49). In *Vibrio vulnificus*, a marine bacterium that causes gastroenteritis when ingested with contaminated seafood, CRP positively regulates the expression of a cytolytic hemolysin and a heme receptor (18, 61). The *Vibrio cholerae* toxin is negatively regulated by CRP through an indirect pathway involving the second regulator, TcpP (47, 70). In contrast, expression of the heat-labile toxin of enterotoxigenic *E. coli* (ETEC) is repressed by CRP when it binds to an operator centered directly over the toxin promoter's −35 hexamer (10). The *Salmonella enterica* serovar Typhimurium Stn enterotoxin is negatively regulated by CRP (51). This is not surprising, because Stn is similar to both cholera and heat-labile toxin.

Adherence to biotic and abiotic surfaces is another important trait of many pathogenic bacteria, and several studies have shown that many types of adhesive pili are CRP regulated. For example, the toxin-coregulated pilus of *V. cholerae* is negatively regulated by CRP (70). The bundle-forming pilus of enteropathogenic *E. coli* may also be repressed by CRP (26, 63). In *Serratia marcescens* and uropathogenic *E. coli*, CRP negatively regulates the expression of type I fimbriae (41, 59). CRP positively regulates the expression of ETEC K99 and 987P pili through direct and indirect pathways, respectively (25, 54). CRP apparently also regulates the expression of other ETEC pili such as CFA/I, CS1, CS2, and CS3 because their expression is subject to catabolite repression (29, 43). In this study, we show that CRP also regulates the expression of the ETEC *tibDBCA* locus, which encodes a glycosylated autotransporter (TibA) that facilitates adherence to mammalian cells, autoaggregation, and biofilm formation (52, 53, 69).

MATERIALS AND METHODS

Strains and plasmids. The relevant features of the *E. coli* strains used in this study are shown in Table 1. The *tibDB* promoter region from −594 through +77, with the numbering relative to the *tibD* ATG start codon, was amplified from the chromosome of ETEC strain H10407 (20, 30) with primers SN879 and SN882. The sequences of oligonucleotide primers are listed in Table 2. The 0.7-kb PCR product was digested with XbaI and EcoRI and then ligated into the same sites

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Miami Miller School of Medicine, P.O. Box 016960 (R-138), Miami, FL 33101. Phone: (305) 243-5317. Fax: (305) 243-7284. E-mail: gmunson@miami.edu.

▽ Published ahead of print on 7 January 2011.

TABLE 1. Bacterial strains

Strain	Relevant features	Reference
H10407	ETEC O78:H11:K80 TibA$^+$ CFA/I$^+$ LT$^+$ STh$^+$ STp$^+$	30
H10407 Δ*crp*	H10407 Δ*crp*	This study
HB101	*recA13 lacY1*	12a
BW25113	Δ*lacZ4787(::rrnB-4)*	22
JW5702	BW25113 *crp*::*kan*	5
JW3778	BW25113 *cyaA*::*kan*	5
GPM1710	BW25113 $attB_{HK022}$::pTibDBLac1 [*tibDBo tibDBp*::*lacZ*]	This study
GPM1713	JW5702 $attB_{HK022}$::pTibDBLac1 [*tibDBo tibDBp*::*lacZ*]	This study
GPM1605	BW25113 $attB_{HK022}$::pTibDBLac2 [*tibDBo1 tibDBp*::*lacZ*]	This study
GPM1607	JW5702 $attB_{HK022}$::pTibDBLac2 [*tibDBo1 tibDBp*::*lacZ*]	This study
GPM1604	BW25113 $attB_{HK022}$::pTibDBLac3 [*tibDBo2 tibDBp*::*lacZ*]	This study
GPM1606	JW5702 $attB_{HK022}$::pTibDBLac3 [*tibDBo2 tibDBp*::*lacZ*]	This study

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence[a]
SN879	.....GCATCTAGAAATGCCGGGCTGGCTG
SN882	.....GACGAATTCGTCTGCGTCTCTCAG
SN884	.....GACGAATCAGTGCAGAGTAACGCG
SN885	.....GACTCTAGATTTTAGCTTTTGTCTGG
SN890	.....GACTCGAGAATTTTTAATTAATTGTTGT TTGC
SN891	.....GTCTCGAGTATAAATCAATAAAACTCTCG
SN892	.....GTCTCGAGTTTATATCAAACAATTTTTAATT AATTG
SN893	.....GACTCGAGAAACTCTCGTATCAAATCCCTTC

[a] Underlined nucleotides indicate primer-template mismatches that add sites for restriction endonucleases.

of the pHKLac1z polylinker to construct pTibDBLac1 [*tibDBp*(−594 to +77)::*lacZ*]. Plasmid pHKLac1z is a promoterless Lac reporter plasmid carrying the *pir*-dependent R6Kγ origin of replication, *aadA* conferring resistance to spectinomycin and streptomycin, and $attP_{HK022}$. Read-through from flanking sequences is prevented by transcriptional terminators located immediately upstream of the polylinker and downstream of *lacZ*. Plasmid pTibDBLac1 served as the PCR template for oligonucleotide-directed mutagenesis of the CRP binding site *tibDBo*. In brief, the plasmid was subjected to inverse PCR with primer pair SN892 and SN893 or SN890 and SN891. The PCR products were digested with XhoI and then circularized with T4 DNA ligase. The resulting binding sites, *tibDBo1* and *tibDBo2*, carry XhoI sites in the promoter-distal and promoter-proximal regions of the CRP binding site, respectively. DNA sequencing confirmed that no other mutations were present in the *tibDB* promoter fragments. To avoid the possibility of PCR errors outside the sequenced regions, the mutagenized promoter fragments were cloned into pHKLac1z as described above. The resulting reporter plasmids, pTibDBLac2 and pTibDBLac3, carry *tibDBo1* and *tibDBo2*, respectively. Lac reporter strains were constructed by integration of reporter plasmids into the chromosomal $attB_{HK022}$ site of BW25113 (22) and its isogenic *crp*::*kan* progeny JW5702 (5) by site-specific recombination with Int_{HK022} (37). Single integrants were verified by colony PCR as previously described (37).

Plasmid pDCRP is an ampicillin-resistant derivative of pBR322 that expresses *crp* from its own promoter (7). Plasmid pDU9 is a Δ*crp* subclone of pDCRP (7). Plasmid pSE186 (39) was constructed by cloning *crp* into the vector pHG165, which confers resistance to ampicillin (71). The complete *tib* locus, *tibDBCA*, was cloned into pHG165 to construct pET109 (28). Plasmid pET142 carries the same 3.6-Kb *tibDBCA141* fragment as pET146 (28) but in pHG165 rather than pACYC184. To construct pGPM1121 (*tibB60CA*); the *tibDB* promoter region, *tibD*, and the first 59 codons of *tibB* were excised from pET109 as a 3.5-Kb BglII fragment.

β-Galactosidase assays. Lac reporter strains were grown aerobically at 37°C in Luria-Bertani (LB) medium (10 g/liter tryptone, 5 g/liter yeast extract, 5 g/liter NaCl) with 30 μg/ml streptomycin, 30 μg/ml spectinomycin, and, as required, 100 μg/ml ampicillin. Cells were harvested from stationary-phase cultures, lysed, and assayed for β-galactosidase activity as previously described (58).

Aggregation assays. Strains BW25113 and JW3778 harboring pET109, pET142, or pGPM1121 were cultured aerobically with agitation to stationary phase at 37°C in LB10 (10 g/liter tryptone, 5 g/liter yeast extract, 0.58 g/liter NaCl) with 100 μg/ml ampicillin. This low-salt medium was used for aggregation assays because we have observed that TibA-dependent aggregation, but not necessarily TibA expression, is progressively inhibited by increasing concentrations of NaCl. High salt concentrations have also been reported to disrupt self-interactions of AIDA-I, another autoaggregating adhesin of diarrheagenic *E. coli* (34). For qualitative comparisons, representative cultures of four or more independent replicates were photographed immediately after vigorous mixing and 3 h after standing at room temperature without agitation. A spectrophotometer was used to measure the absorbance at 600 nm of aliquots from the upper portion of selected cultures. The aggregation factor was calculated as the difference between the mixed and static absorbance readings.

DNase I footprinting. CRP was purified to 89% purity, as determined by lab-on-chip analysis (Agilent Technologies), on an immobilized metal ion affinity column as previously described (10, 74). For DNase I footprinting of the coding strand, a DNA template from −334 through +57 was produced by PCR with primer SN885 and ^{32}P-end-labeled primer SN882 (Table 2). For the noncoding strand, a PCR product from −576 through −70 was generated with primer SN879 and ^{32}P-end-labeled primer SN884. The PCR products, whose numbering is relative to the *tibD* initiating codon, were purified on nondenaturing acrylamide gels as previously described (60). Following their purification, the DNA templates were equilibrated with or without CRP for 30 min at 37°C in 10 mM Tris-Cl (pH 7.6), 50 mM KCl, 1 mM dithiothreitol, 2 ng/μl poly(dI-dC), 0.4 mM $MgCl_2$, 0.2 mM $CaCl_2$, 100 μg/ml bovine serum albumin, 0.5 mM cAMP. Once equilibrated, DNA templates were digested with DNase I at a final concentration of 100 ng/μl for 1 min at 37°C. Cleavage reactions were terminated by the addition of approximately 10 volumes of 570 mM NH_4 acetate, 50 μg/ml tRNA, 80% (vol/vol) ethanol. DNA was then precipitated on dry ice, washed with 70% (vol/vol) ethanol, and suspended in 4 μl of 80% (vol/vol) formamide, 50 mM Tris-borate (pH 8.3), 1 mM EDTA, 0.1% (wt/vol) xylene cyanol, 0.1% (wt/vol) bromophenol blue. After denaturation at 85°C for 5 min, aliquots were separated on sequencing gels alongside TC and GA sequence ladders (57). Gels of primer extension and footprinting reactions were exposed to phosphor screens (Bio-Rad Laboratories), which were then scanned with a phosphorimager (GE Healthcare). Digital densitometry was done using ImageJ (1) running under Mac OS X (Apple Inc.).

Primer extension. Total RNA was harvested from Lac reporter strains GPM1710, GPM1713, GPM1604, GPM1605, GPM1606, and GPM1607 grown to mid-log phase in LB medium at 37°C as described elsewhere (11). ^{32}P-end-labeled primer SN884 was combined with 15 to 40 μg of total RNA, and the solution was heated for 5 min at 65°C and then cooled on ice for 2 min. The annealed primer was then extended with SuperScript III reverse transcriptase according to the manufacturer's protocol (Invitrogen). Aliquots of each reaction mixture were separated on sequencing gels alongside dideoxy chain-terminated sequencing ladders (66).

***In silico* analyses.** Mauve (21) was used to align the chromosomes of ETEC strain H10407 (GenBank accession no. FN649414) and K-12 strain MG1655 (accession no. U00096) (9, 20). A data set and nucleotide frequency table of 139 aligned CRP binding sites were downloaded from the RegTransBase database (44) (http://regtransbase.lbl.gov/). The frequency table was then used to calculate a position-weighted matrix as described previously (62). The position-weighted matrix was then used by our own software (DNAEntropy) to search for and rank putative CRP binding sites within select regions of the H10407 chromosome.

Membrane fractionation. Outer membrane fractions were isolated as previously described (28). Briefly, bacteria were cultured aerobically at 37°C in LB medium to late log phase, harvested by centrifugation, and then lysed by two passages through a French press. Inner and outer membranes were separated by sucrose density ultracentrifugation (67). The protein concentration of membrane fractions was determined by the Bradford method (13) with reagents purchased from Bio-Rad Laboratories.

Protein electrophoresis. Electrophoresis of membrane fractions was performed under denaturing conditions by the method of Laemmli (48). Gels were stained for proteins with Coomassie blue or for glycoprotein as described below. For immunoblotting, proteins separated by electrophoresis were transferred to nitrocellulose and then blocked with casein filler solution (10 mM Tris-buffered

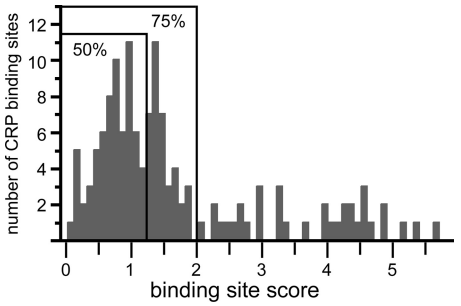

FIG. 1. *In silico* identification of a CRP binding site upstream of *tibDBCA*. Histogram of characterized CRP binding sites. A position-weighted matrix was calculated from a frequency table derived from 139 aligned CRP binding sites. The matrix was then used to score each of the 139 binding sites as the product of the Shannon redundancy index and the Berg-von Hippel function. Low scores indicate a better fit to the collection of known binding sites than high scores. Bound regions enclose 50% and 75% of the 139 binding sites.

saline [pH 7.4], 2% [wt/vol] casein, 0.1% [wt/vol] sodium azide). Blocked filters were incubated for 1 h with a 1:200 dilution (in casein filler) of rabbit polyclonal anti-TibA antibody (52), washed in Tris-buffered saline containing 0.05% (vol/vol) Triton X-100, incubated for 1 h with alkaline phosphatase-conjugated protein A (Sigma) (1:2,000 dilution in casein filler), rewashed, and then detected with nitroblue tetrazolium chloride–5-bromo-4-chloro-3-indolyl phosphate toluidine salt (NBT-BCIP; Roche).

Detection of glycoproteins. Glycoproteins were detected on nitrocellulose membranes using method B of the digoxigenin (DIG) glycan detection kit according to the manufacturer's recommendations (Roche). Briefly, proteins were separated by SDS-PAGE and then transferred to nitrocellulose. Membranes were washed in phosphate-buffered saline (PBS; 50 mM potassium phosphate [pH 6.5], 150 mM NaCl), and carbohydrates were oxidized with sodium metaperiodate. Oxidized carbohydrates were labeled with DIG-conjugated hydrazide. DIG-labeled proteins were visualized using alkaline phosphatase-conjugated anti-DIG antibodies and then detected with NBT-BCIP.

Digital images. Digital images were cropped and scaled using Canvas X (ACD Systems) running under Mac OS X (Apple Inc.). The same software was also used to adjust brightness and contrast uniformly across digital images as necessary for visual clarity.

RESULTS

Identification of a CRP binding site upstream of *tibDB*. CRP regulates the expression of several ETEC virulence factors, including enterotoxins and certain types of adhesive pili (10, 25, 29, 43, 54). Thus, we reasoned that the further elucidation of the CRP regulon in ETEC could advance our understanding of this pathogen. Since microarrays containing an ETEC genome were not available for this study, we sought to identify CRP-regulated genes using a bioinformatics approach. To do this, we used a frequency table compiled from 139 known CRP binding sites to calculate a position-weighted matrix. The matrix was then used to search the genome of ETEC strain H10407 for potential CRP binding sites. Putative binding sites were ranked by the product of the Shannon redundancy index and Berg-von Hippel function (62). To limit the identification of false positives, we also restricted our analysis to putative sites ranking below a Shannon redundancy index of 2.0 because low values indicate a better fit to the data set of known CRP binding sites than high values. Although this cutoff value is arbitrary, 75% of the 139 characterized CRP binding sites in the RegTransBase database score below this threshold (Fig. 1). Since CRP binding sites have already been extensively characterized in K-12 strains, we further restricted our analysis to pathogen-specific regions of the chromosome. These unique regions were identified by aligning the H10407 and K-12 chromosomes (35, 36, 45). Among the putative CRP binding sites that we identified, one is located within a 16-Kb insertion at min 44.8 relative to the MG1655 chromosome. Features flanking this insertion include the CP4-44 cryptic prophage and *sbmC* (http://www.ecogene.org/). The putative CRP binding site, *tibDBo*, is located within a 372-bp intergenic region upstream of *tibDB* (Fig. 2A). Relative to the 139 binding sites that define the position-weighted matrix, *tibDBo* ranks within the 50th percentile with a score of 1.1 (Fig. 1).

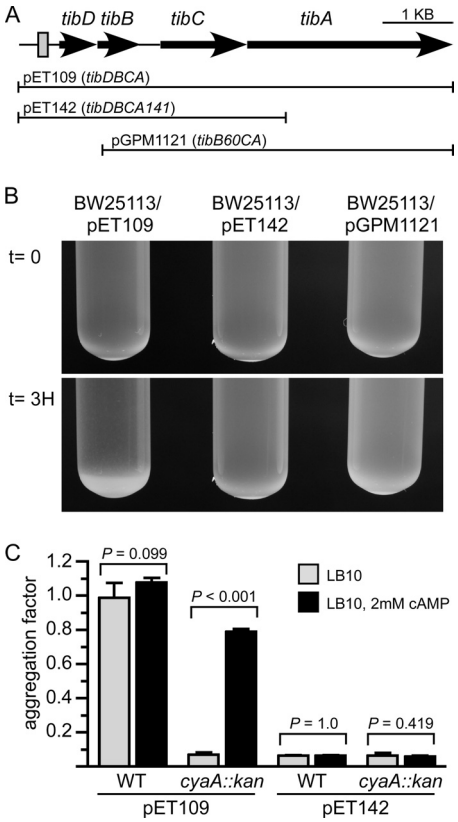

FIG. 2. TibA-dependent aggregation. (A) Graphic depicting the chromosomal *tib* locus of ETEC strain H10407. The predicted CRP binding site upstream of *tibDB* is represented by a shaded rectangle. Solid lines represent regions of the *tib* locus carried by plasmids pET109 (*tibDBCA*), pET142 (*tibDBCA141*), and pGPM1121 (*tibB60CA*). (B) Representative stationary-phase cultures photographed immediately after mixing (t = 0) and 3 h after standing without agitation (t = 3H). (C) The absorbance (600 nm) of the upper fraction from stationary-phase cultures was read immediately after mixing and after the cultures were allowed to stand for 3 h without agitation. The difference between the two absorbance readings was used as the aggregation factor. Error bars represent the standard deviations of the means ($n = 4$). *P* values were calculated using the Student *t* test. WT, wild-type K-12 strain BW25113; *cyaA*::*kan*, K-12 strain JW3778.

TibA expression is dependent upon CRP. Since bacteria expressing TibA autoaggregate and settle out of solution (69), we evaluated TibA expression by monitoring the aggregation of K-12 strains that were transformed with various plasmids. We observed that BW25113 aggregated and settled out of solution more rapidly when it harbored pET109 (*tibDBCA*)

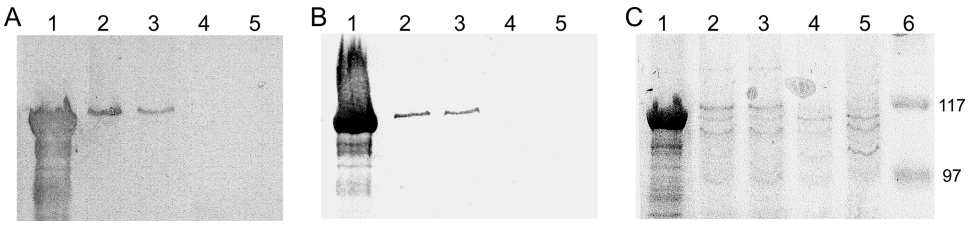

FIG. 3. TibA expression in H10407 and H10407 Δ*crp*. Outer membranes were purified from the following strains (by lane): 1, HB101/pET109 (*tibDBCA*); 2, H10407; 3, H10407 Δ*crp*/pSE186 (*crp*$^+$); 4, H10407 Δ*crp*/pHG165 (vector); and 5, H10407 Δ*crp*. Plasmid pHG165 is the vector control for both pET109 and pSE186. Outer membrane fractions were then separated by SDS-PAGE and subjected to immunoblotting with anti-TibA polyclonal antibody (A), glycoprotein staining (B), or Coomassie blue staining (C). As indicated, lane 6 contains molecular mass standards of 117 and 97 kDa. TibA has an expected molecular mass of 98 kDa, which is increase to ~104 kDa by glycosylation (69).

than when it harbored pET142 (*tibDBCA141*) (Fig. 2B). This result was expected and confirms that this assay reflects TibA-dependent aggregation, because *tibA141* is a deletion of the *tibA* 3′ coding region (Fig. 2A). In addition to *tibA*, aggregation was found to be dependent upon *tibDB* and/or the *tibDB* promoter region because BW25113/pGPM1121 (*tibB60CA*) failed to aggregate (Fig. 2B). This result also demonstrates that a *tib*-encoded promoter is required for TibA expression, as opposed to a vector-encoded promoter, because pGPM1121 (*tibB60CA*) was constructed by deletion of an internal *tib* fragment from pET109 (*tibDBCA*) without disruption of flanking vector sequences. In addition, this *tib*-encoded promoter must lie upstream of *tibB*.

Having established that aggregation is an indicator of TibA expression from a *tib*-encoded promoter, we next compared aggregation of a *cyaA*::*kan* strain to that of an isogenic wild-type strain. Unlike the wild-type strain harboring pET109 (*tibDBCA*), which readily aggregated, aggregation was abolished in the *cyaA*::*kan* strain unless cAMP was provided exogenously (Fig. 2C). Since CRP requires cAMP for its activity, these results suggest that CRP is a positive regulator of TibA expression. As expected, strains harboring pET142 (*tibDBCA141*) failed to aggregate regardless of genetic background or exogenous cAMP.

In addition to aggregation assays with K-12 strains, we also evaluated the expression of TibA in ETEC strain H10407 and its isogenic H10407 Δ*crp* derivative. In agreement with our aggregation assays, we found by Western blotting that TibA expression was abolished in H10407 Δ*crp* (Fig. 3A). Transformation of H10407 Δ*crp* with the CRP expression plasmid pSE186 restored TibA expression. In contrast, TibA was not expressed when H10407 Δ*crp* was transformed with the vector control pHG165. Since TibA is glycosylated by TibC, Western blot results were mirrored by glycoprotein staining (Fig. 3B). Taken together, the results of our aggregation assays, Western blotting, and glycoprotein staining demonstrate that CRP is required for the expression of TibA.

CRP binds to *tibDBo in vitro*. As expected from its relatively low score, *in vitro* DNase I footprinting confirmed that CRP binds to the site predicted by *in silico* analysis (Fig. 4). With respect to the *tibD* start codon, CRP protected a region from −212 through −188 on the coding strand and from −218 through −187 on the noncoding strand. CRP-dependent DNase I hypersensitive sites were also observed at −204 and −195 on the coding strand and −209 and −199 on the noncoding strand. These hypersensitive sites may indicate a distortion of the DNA helix, which is expected because CRP has been shown to bend DNA by as much as 90 degrees (68, 72). An additional hypersensitive site was observed at −248 on the coding strand. Curiously, this site lies approximately three helical turns upstream of the DNase I footprint of bound CRP. Since hypersensitivity is often visually apparent before protection, the hypersensitive site at −248 may indicate the presence of an unsaturated low-affinity CRP binding site. This phenomenon is evident on the noncoding strand at position −209, which displays hypersensitivity at 15 nM CRP in the absence of substantial protection of other bases within the site (Fig. 4).

CRP activates the *tibDB* promoter. To evaluate the biological relevance of the CRP binding site, we integrated a *tibDBp*::*lacZ* reporter plasmid into the chromosomal $attB_{HK022}$ site of wild-type and *crp*::*kan* K-12 strains. Our analysis revealed that expression of β-galactosidase was 7-fold lower in the *crp*::*kan* reporter strain than in the wild-type reporter strain (Fig. 5A). Expression of β-galactosidase was restored to wild-type levels when CRP was provided in *trans* to the *crp*::*kan* mutant. As expected, the vector control plasmid pDU9 had no effect in the same genetic background. We also evaluated the effects of point mutations within binding site *tibDBo* (Fig. 5B). Mutagenized binding site *tibDBo1* carries six mutations in the promoter-distal portion of the CRP binding site, while *tibDBo2* carries five mutations in the promoter-proximal portion (Fig. 6). We found that *tibDBo1* and *tibDBo2* abolish CRP's ability to activate *tibDBp* (Fig. 5B). Thus, these results demonstrate that occupancy of *tibDBo*, which was predicted *in silico* and subsequently confirmed by DNase I footprinting, results in CRP-dependent activation of the *tibDB* promoter.

Identification of CRP-dependent transcription start sites. After determining that CRP activates *tibDBp*, we used primer extension to map the promoter's CRP-dependent transcription start sites (Fig. 6A). P2, the most abundant primer extension product by digital densitometry, mapped to an adenosine at −142 relative to the *tibD* start codon (Fig. 6B). A less pronounced primer extension product, P3, mapped to an adenosine at −139, followed by a minor product, P1, that corresponds to an adenosine at −151. Although only one representative reaction is shown, the relative intensities of the three bands (P2 > P3 > P1) was reproducible. In addition, all three bands are CRP dependent because they were not observed with RNA harvested from *crp*::*kan* strains (Fig. 6A). The CRP binding site mutations *tibDBo1* and *tibDBo2* also abolished the three primer extension products, even in the *crp*$^+$ background (Fig. 6A). However, β-galactosidase assays demonstrate that the

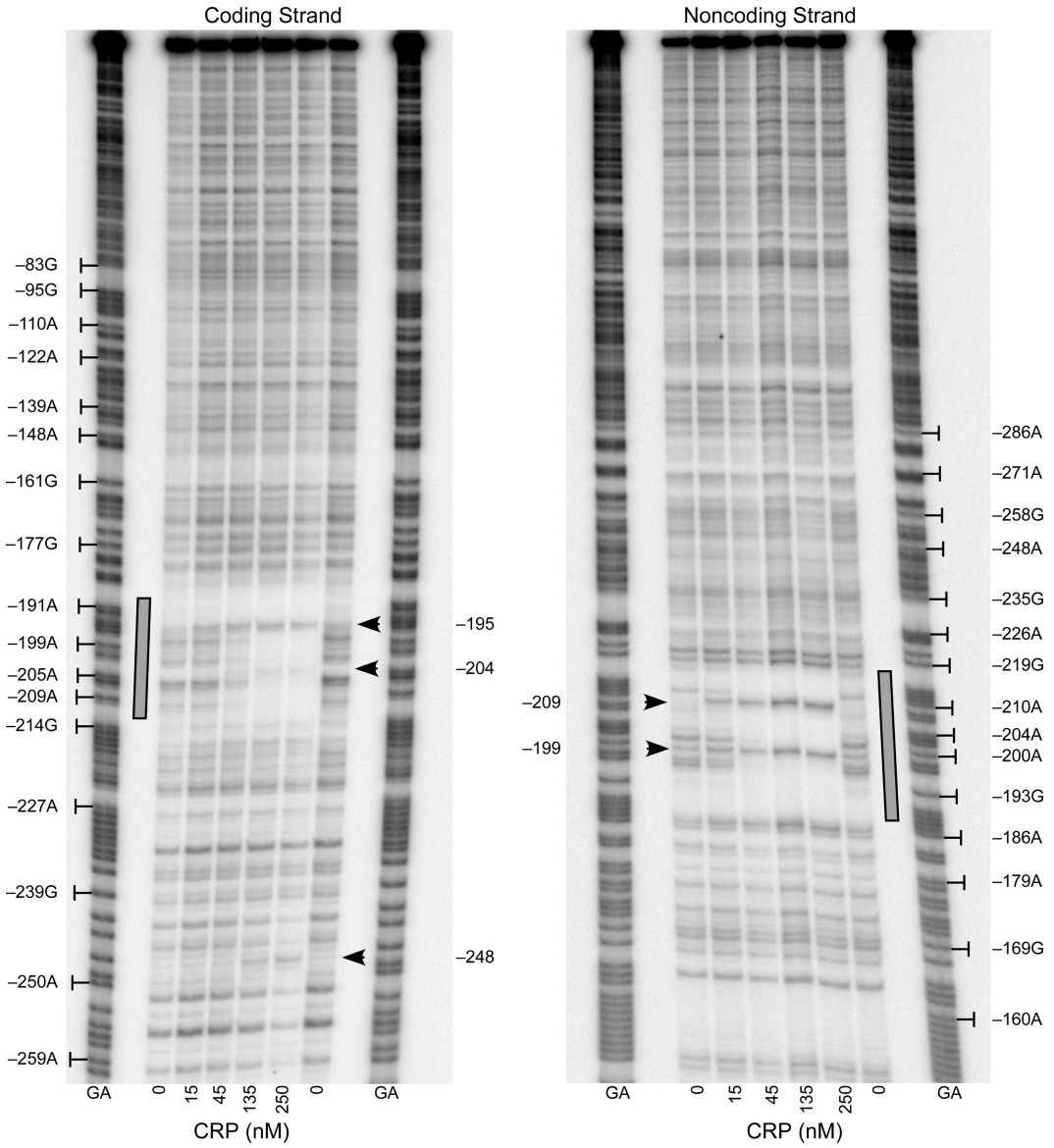

FIG. 4. CRP binds to a site upstream of *tibDB*. DNase I footprints of CRP bound to coding and noncoding strands of *tibDBp* are shown. The CRP binding site, *tibDBo*, is highlighted by shaded rectangles. Arrowheads indicate CRP-dependent DNase I hypersensitive sites. Numbering is relative to the *tibD* start codon. GA, Maxam-Gilbert sequence ladders.

tibDB promoter is not completely silenced by these mutations or in the *crp*::*kan* background. Thus, the absence of primer extension products does not indicate a complete absence of transcription. It does, however, indicate that the transcripts are below the limits of detection by primer extension. In addition to the three *bona fide* primer extension products, we also observed a band near position −131 that was present in all primer extension reactions, including the dideoxy sequencing reactions, which utilized the same primer (Fig. 6A). This observation was confirmed by densitometric analyses and indicates that the band near −131 is a primer or gel running artifact.

DISCUSSION

In this study, we have shown that CRP activates the *tibDB* promoter by occupying a binding site upstream of *tibD*. We have also identified three CRP-dependent primer extension products in the *tibDB* promoter region. The CRP binding site is centered 59.5 bp upstream of P2, the most abundant primer extension product, and 62.5 bp upstream of P3, the second most abundant product (Fig. 6B). The distance of these transcription start sites to the CRP binding site suggests that *tibDBp* is primarily a class I promoter (14). With regard to P1, the minor primer extension product, the 50.5 bp between the CRP binding site center and the transcription start site is ambiguous and will require additional experimentation before P1 can be classified with confidence. Additional experimentation will also be required to identify the −10 and −35 hexamers of each promoter. Nevertheless, it is clear from these studies that CRP binds to *tibDBo*, activates the *tibDB* promoter, and is required for TibA expression. Furthermore there is no evidence of an additional CRP-dependent promoter within or downstream of

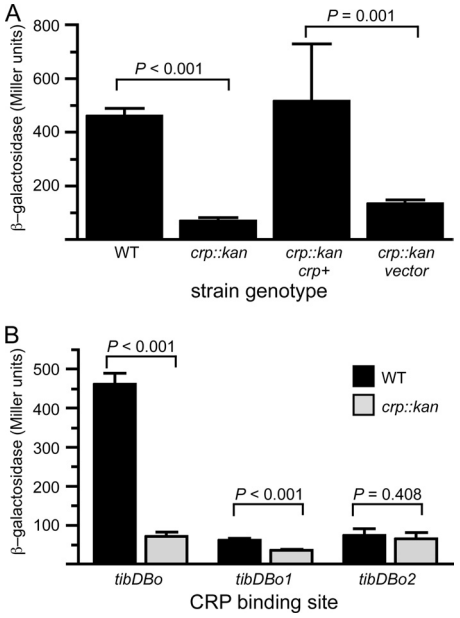

FIG. 5. The *tibDB* promoter is activated by CRP. β-Galactosidase activities of *tibDBp*::*lacZ* reporters integrated into the chromosomal $attB_{HK022}$ site of wild-type strain BW25113 or *crp*::*kan* strain JW5702 are shown. Assays were conducted in triplicate on two successive days ($n = 6$), and error bars show the standard deviations of the means. *P* values were calculated using the Student *t* test. (A) Reporter strains GPM1710 (WT) and GPM1713 (*crp*::*kan*) carrying $attB_{HK022}$::pTibDBLac1 (*tibDBo*). For complementation assays, GPM1713 was transformed with pDCRP (crp^+) or pDU9 (vector). (B) *tibDBp* activity in wild-type (WT) and *crp*::*kan* reporter strains carrying the natural CRP binding site *tibDBo* or the mutagenized binding sites *tibDBo1* and *tibDBo2*.

tibB, because a strain carrying pGPM1121 (*tibB60CA*) did not display TibA-dependent autoaggregation.

Transposon mutagenesis and subcloning of the *tib* locus has shown that TibB is required for the expression of TibA (28). TibA is a 104-kDa protein that utilizes a carboxy-terminal autotransporter domain to reach the outer membrane, where it functions as an adhesin to mammalian cells (38, 53). TibA also mediates bacterial aggregation and biofilm formation (69). Adherence to mammalian cells, but not aggregation and biofilm formation, is dependent upon glycosylation of TibA by TibC (28, 69). Although *tibDB* are naturally located in *cis* with *tibCA* (Fig. 2A), TibA is also expressed when *tibDB* are provided in *trans* (28). Moreover, TibB and/or TibD apparently function at the level of transcription, because they are not required for the expression or membrane localization of TibA when it is expressed from a heterologous promoter such as *lacp*. TibB is probably a DNA binding protein because it contains a LuxR-like DNA binding helix-turn-helix motif near its carboxy terminus. We have also identified an 18-bp spaced inverted repeat (CAACGACTAAAGTCGTTG) centered 196 bp upstream of *tibC* within the intergenic region between *tibDB* and *tibCA* (Fig. 2A). This spaced inverted repeat may function as a TibB binding site, because other proteins with LuxR DNA binding motifs have been shown to bind spaced inverted repeats (23, 55, 73).

The translation of *tibB* is likely coupled to that of *tibD* because the two genes overlap by 4 bp. The *tibD* gene encodes a 20.4-kDa protein that is predicted to be cytoplasmic because it lacks a signal peptide and transmembrane helices. Residues

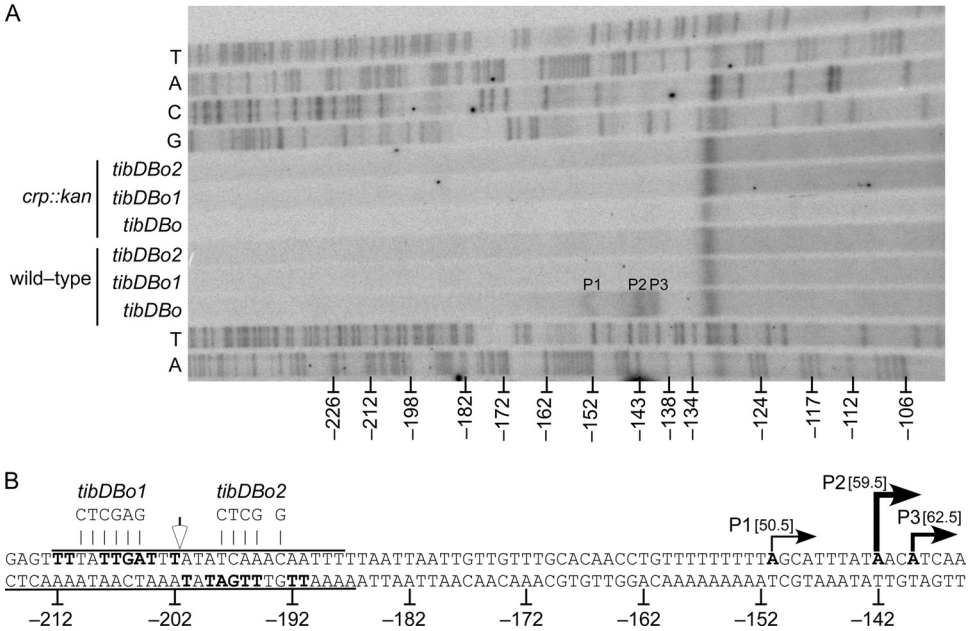

FIG. 6. Identification of CRP-dependent transcription start sites. Numbering is relative to the *tibD* initiating codon. (A) Primer extension was used to map the transcription start sites of *tibDBp* in wild-type and *crp*::*kan* reporter strains carrying the natural CRP binding site, *tibDBo*, or the mutagenized binding sites, *tibDBo1* and *tibDBo2*. Lanes T, A, C, and G contain dideoxy chain-terminated sequencing ladders produced with the same primer as the primer extension reactions. The band running across all lanes near −131 is an artifact. (B) Sequences of the *tibDB* promoter region and the mutagenized binding sites *tibDBo1* and *tibDBo2*. Overlines and underlines indicate the extent of CRP DNase I footprints. The inverted repeat within the binding site is shown in bold. Filled arrows and bold type denote the positions of the three transcription start sites. The weight of each line/arrow reflects the relative proportions of each primer extension product. The distance between the center of the CRP binding site (unfilled arrow) and each transcription start site is shown in brackets.

59 to 80 are predicted to form a helix-turn-helix motif (19), and this motif is contained within a larger region with similarity to the PF07180 (DUF1401) superfamily, whose members include GrlA and CaiF (32). GrlA is encoded within, and positively regulates other genes within, the locus of enterocyte attachment and effacement of enterohemorrhagic *E. coli*, enteropathogenic *E. coli*, and *Citrobacter rodentium* (6, 27, 40, 64). Additionally, GrlA has been shown to positively regulate the expression of enterohemolysin in enterohemorrhagic *E. coli* (65). CaiF is an activator of the *E. coli caiTABCDE* and *fixABCX* operons, which are required for carnitine metabolism under anaerobic conditions (15). Although this suggests that TibD is a transcription factor, it is not yet known if TibD is required for *tibCA* expression, because Tn*5* insertions within *tibD* undoubtedly also disrupted TibB expression. Nevertheless, our results, when combined with previous studies that have shown that TibB is required for TibA expression (28), indicate that CRP positively regulates the expression of *tibCA* through an indirect pathway involving TibB and possibly TibD. The expression of the TibA-like AIDA-I adhesin has also been reported to be subject to catabolite repression (8). However, it is not yet known if this is a direct or indirect effect.

CRP has also been shown to regulate the expression of other ETEC virulence factors. For example, it has recently been shown that CRP represses the expression of the heat-labile toxin (10). Unlike TibA, which is regulated through an indirect pathway, CRP regulates the expression of the heat-labile toxin by binding to a site centered over the −35 hexamer of the toxin's promoter. CRP also regulates the expression of heat-stable toxins (2, 3, 10, 17, 56). Adhesive pili, such as CFA/I, CS1, CS2, and CS3, have also been reported to be subject to catabolite repression (29, 43). For CS1, CS2, and CS3 pili, the effect is produced indirectly through CRP-dependent expression of the regulator Rns (M. D. Bodero and G. P. Munson, unpublished). Thus, it is now clear that nearly all of the characterized ETEC virulence factors are regulated by CRP through direct or indirect pathways. Since the activity of CRP is inhibited when sugars such as glucose suppress the synthesis of cAMP, the pathogen may use the lumenal concentration of glucose as a cue to differentially express its virulence factors as it moves through the small intestine, as has been suggested previously (10, 25). CRP-repressed virulence factors, such as heat-labile toxin, may be expressed predominately in the duodenum, where the concentration of glucose is relatively high compared to that in the lower sections of the small intestine (31). As the pathogen travels toward the ileum, catabolite repression would be relieved as glucose and other monosaccharides are progressively absorbed by the small intestine. As a result, CRP-dependent adherence factors such as TibA and certain types of pili may not be fully expressed until the pathogen reaches the lower sections of the small intestine. This model for the spatial distribution of ETEC virulence factors is consistent with a murine model of colonization that has shown preferential colonization of the ileum by H10407 (4).

ACKNOWLEDGMENTS

We thank Stephen Busby, Susan Egan, and Alan Wolfe for providing strains and plasmids.

This work was supported by NIAID grant number 057648.

The contents of this publication are solely the responsibility of the authors and do not necessarily represent the official views of the NIH.

REFERENCES

1. **Abramoff, M. D., P. J. Magalhaes, and S. J. Ram.** 2004. Image processing with image. J. Biophotonics Int. **11:**36–41.
2. **Alderete, J. F., and D. C. Robertson.** 1977. Nutrition and enterotoxin synthesis by enterotoxigenic strains of *Escherichia coli*: defined medium for production of heat-stable enterotoxin. Infect. Immun. **15:**781–788.
3. **Alderete, J. F., and D. C. Robertson.** 1977. Repression of heat-stable enterotoxin synthesis in enterotoxigenic *Escherichia coli*. Infect. Immun. **17:**629–633.
4. **Allen, K., M. Randolph, and J. Fleckenstein.** 2006. Importance of heat-labile enterotoxin in colonization of the adult mouse small intestine by human enterotoxigenic *Escherichia coli* strains. Infect. Immun. **74:**869–875.
5. **Baba, T., et al.** 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol. Syst. Biol. **2:**1–11.
6. **Barba, J., et al.** 2005. A positive regulatory loop controls expression of the locus of enterocyte effacement-encoded regulators Ler and GrlA. J. Bacteriol. **187:**7918–7930.
7. **Bell, A., et al.** 1990. Mutations that alter the ability of the *Escherichia coli* cyclic AMP receptor protein to activate transcription. Nucleic Acids Res. **18:**7243–7250.
8. **Berthiaume, F., M. F. Leblond, J. Harel, and M. Mourez.** 2010. Growth-phase-dependent expression of the operon coding for the glycosylated autotransporter adhesin AIDA-I of pathogenic *Escherichia coli*. FEMS Microbiol. Lett. **311:**176–184.
9. **Blattner, F. R., et al.** 1997. The complete genome sequence of Escherichia coli K-12. Science **277:**1453–1462.
10. **Bodero, M., and G. Munson.** 2009. Cyclic AMP receptor protein-dependent repression of heat-labile enterotoxin. Infect. Immun. **77:**791–798.
11. **Bodero, M., M. Pilonieta, and G. Munson.** 2007. Repression of the inner membrane lipoprotein NlpA by Rns in enterotoxigenic *Escherichia coli*. J. Bacteriol. **189:**1627–1632.
12. **Botsford, J., and J. Harman.** 1992. Cyclic AMP in prokaryotes. Microbiol. Mol. Biol. Rev. **56:**100–122.

12a. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41:**459–472.

13. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
14. **Browning, D., and S. Busby.** 2004. The regulation of bacterial transcription initiation. Nat. Rev. Microbiol. **2:**57–65.
15. **Buchet, A., W. Nasser, K. Eichler, and M. Mandrand-Berthelot.** 1999. Positive co-regulation of the *Escherichia col*i carnitine pathway *cai* and *fix* operons by CRP and the CaiF activator. Mol. Microbiol. **34:**562–575.
16. **Busby, S., and R. Ebright.** 1999. Transcription activation by catabolite activator protein (CAP). J. Mol. Biol. **293:**199–213.
17. **Busque, P., A. Letellier, J. Harel, and J. D. Dubreuil.** 1995. Production of *Escherichia coli* STb enterotoxin is subject to catabolite repression. Microbiology **141:**1621–1627.
18. **Choi, H. K., et al.** 2002. Promoter analysis and regulatory characteristics of *vvhBA* encoding cytolytic hemolysin of *Vibrio vulnificus*. J. Biol. Chem. **277:**47292–47299.
19. **Combet, C., C. Blanchet, C. Geourjon, and G. Deléage.** 2000. NPS@: network protein sequence analysis. Trends Biochem. Sci. **25:**147–150.
20. **Crossman, L. C., et al.** 2010. A commensal gone bad: complete genome sequence of the prototypical enterotoxigenic *Escherichia coli* strain H10407. J. Bacteriol. **192:**5822–5831.
21. **Darling, A. C. E., B. Mau, F. R. Blattner, and N. T. Perna.** 2004. Mauve: multiple alignment of conserved genomic sequence with rearrangements. Genome Res. **14:**1394–1403.
22. **Datsenko, K., and B. Wanner.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. U. S. A. **97:**6640–6645.
23. **Devine, J. H., G. S. Shadel, and T. O. Baldwin.** 1989. Identification of the operator of the *lux* regulon from the *Vibrio fischeri* strain ATCC 7744. Proc. Natl. Acad. Sci. U. S. A. **86:**5688–5692.
24. **Ebright, R.** 1993. Transcription activation at class I CAP-dependent promoters. Mol. Microbiol. **8:**797–802.
25. **Edwards, R., and D. Schifferli.** 1997. Differential regulation of *fasA* and *fasH* expression of *Escherichia coli* 987 P fimbriae by enviromental cues. Mol. Microbiol. **25:**797–809.
26. **Edwards, R. A., and J. L. Puente.** 1998. Fimbrial expression in enteric bacteria: a critical step in intestinal pathogenesis. Trends Microbiol. **6:**282–287.
27. **Elliott, S. J., et al.** 1999. Identification of CesT, a chaperone for the type III secretion of Tir in enteropathogenic *Escherichia coli*. Mol. Microbiol. **33:**1176–1189.
28. **Elsinghorst, E. A., and J. A. Weitz.** 1994. Epithelial cell invasion and adherence directed by the enterotoxigenic *Escherichia coli tib* locus is associated

with a 104-kilodalton outer membrane protein. Infect. Immun. **62:**3463–3471.
29. **Evans, D., D. Evans, T. Karjalainen, and C. Lee.** 1991. Production of colonization factor antigen II of enterotoxigenic *Escherichia coli* is subject to catabolite repression. Curr. Microbiol. **23:**71–74.
30. **Evans, D., R. Silver, D. Evans, Jr., D. Chase, and S. Gorbach.** 1975. Plasmid-controlled colonization factor associated with virulence in *Esherichia coli* enterotoxigenic for humans. Infect. Immun. **12:**656.
31. **Ferraris, R., S. Yasharpour, K. Lloyd, R. Mirzayan, and J. Diamond.** 1990. Luminal glucose concentrations in the gut under normal conditions. Am. J. Physiol. **259:**G822–837.
32. **Finn, R. D., et al.** 2010. The Pfam protein families database. Nucleic Acids Res. **38:**D211–222.
33. **Gama-Castro, S., et al.** 2008. RegulonDB (version 6.0): gene regulation model of *Escherichia coli* K-12 beyond transcription, active (experimental) annotated promoters and Textpresso navigation. Nucleic Acids Res. **36:**D120–D124.
34. **Girard, V., et al.** 2010. Conformation change in a self-recognizing autotransporter modulates bacterial cell-cell interaction. J. Biol. Chem. **285:**10616–10626.
35. **Gosset, G., Z. Zhang, S. Nayyar, W. A. Cuevas, and M. H. Saier.** 2004. Transcriptome analysis of Crp-dependent catabolite control of gene expression in *Escherichia coli*. J. Bacteriol. **186:**3516–3524.
36. **Grainger, D. C., D. Hurd, M. Harrison, J. Holdstock, and S. J. W. Busby.** 2005. Studies of the distribution of *Escherichia coli* cAMP-receptor protein and RNA polymerase along the *E. coli* chromosome. Proc. Natl. Acad. Sci. U. S. A. **102:**17693–17698.
37. **Haldimann, A., and B. Wanner.** 2001. Conditional-replication, integration, excision, and retrieval plasmid-host systems for gene structure-function studies of bacteria. J. Bacteriol. **183:**6384–6393.
38. **Henderson, I., and J. Nataro.** 2001. Virulence functions of autotransporter proteins. Infect. Immun. **69:**1231–1243.
39. **Holcroft, C. C., and S. M. Egan.** 2000. Roles of cyclic AMP receptor protein and the carboxyl-terminal domain of the alpha subunit in transcription activation of the *Escherichia coli rhaBAD* operon. J. Bacteriol. **182:**3529–3535.
40. **Iyoda, S., et al.** 2006. The GrlR-GrlA regulatory system coordinately controls the expression of flagellar and LEE-encoded type III protein secretion systems in enterohemorrhagic *Escherichia coli*. J. Bacteriol. **188:**5682–5692.
41. **Kalivoda, E. J., N. A. Stella, D. M. O'Dee, G. J. Nau, and R. M. Q. Shanks.** 2008. The cyclic AMP-dependent catabolite repression system of *Serratia marcescens* mediates biofilm formation through regulation of type 1 fimbriae. Appl. Environ. Microbiol. **74:**3461–3470.
42. **Kanack, K. J., L. J. Runyen-Janecky, E. P. Ferrell, S.-J. Suh, and S. E. H. West.** 2006. Characterization of DNA-binding specificity and analysis of binding sites of the *Pseudomonas aeruginosa* global regulator, Vfr, a homologue of the *Escherichia coli* cAMP receptor protein. Microbiology **152:**3485–3496.
43. **Karjalainen, T., D. Evans, and D. Evans.** 1991. Catabolite repression of the colonization factor antigen I (CFA/I) operon of *Escherichia coli*. Curr. Microbiol. **23:**307–313.
44. **Kazakov, A. E., et al.** 2007. RegTransBase—a database of regulatory sequences and interactions in a wide range of prokaryotic genomes. Nucleic Acids Res. **35:**D407–412.
45. **Khankal, R., J. W. Chin, D. Ghosh, and P. C. Cirino.** 2009. Transcriptional effects of CRP* expression in *Escherichia coli*. J. Biol. Eng. **3:**13.
46. **Kim, T.-J., et al.** 2007. Direct transcriptional control of the plasminogen activator gene of *Yersinia pestis* by the cyclic AMP receptor protein. J. Bacteriol. **189:**8890–8900.
47. **Kovacikova, G., and K. Skorupski.** 2001. Overlapping binding sites for the virulence gene regulators AphA, AphB and cAMP-CRP at the *Vibrio cholerae tcpPH* promoter. Mol. Microbiol. **41:**393–407.
48. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
49. **Lathem, W. W., P. A. Price, V. L. Miller, and W. E. Goldman.** 2007. A plasminogen-activating protease specifically controls the development of primary pneumonic plague. Science **315:**509–513.
50. **Lawson, C. L., et al.** 2004. Catabolite activator protein: DNA binding and transcription activation. Curr. Opin. Struct. Biol. **14:**10–20.
51. **Lim, S., et al.** 2003. Molecular analysis of *Salmonella* enterotoxin gene expression. J. Microbiol. Biotechnol. **13:**598–606.
52. **Lindenthal, C., and E. A. Elsinghorst.** 2001. Enterotoxigenic *Escherichia coli* TibA glycoprotein adheres to human intestine epithelial cells. Infect. Immun. **69:**52–57.
53. **Lindenthal, C., and E. A. Elsinghorst.** 1999. Identification of a glycoprotein produced by enterotoxigenic *Escherichia coli*. Infect. Immun. **67:**4084–4091.
54. **Lo-Tseng, T., J. Lee, and R. E. Isaacson.** 1997. Regulators of *Escherichia coli* K99 region 1 genes. Adv. Exp. Med. Biol. **412:**303–310.
55. **Maris, A. E., et al.** 2002. Dimerization allows DNA target site recognition by the NarL response regulator. Nat. Struct. Biol. **9:**771–778.
56. **Martinez-Cadena, M., L. Guzman-Verduzco, H. Stieglitz, and Y. Kupersztoch-Portnoy.** 1981. Catabolite repression of *Escherichia coli* heat-stable enterotoxin activity. J. Bacteriol. **145:**722–728.
57. **Maxam, A. M., and W. Gilbert.** 1977. A new method for sequencing DNA. Proc. Natl. Acad. Sci. U. S. A. **74:**560–564.
58. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
59. **Müller, C., A. Åberg, and J. Straseviçiene.** 2009. Type 1 fimbriae, a colonization factor of uropathogenic *Escherichia coli*, are controlled by the metabolic sensor CRP-cAMP. PLoS Pathog. **5:**1–14.
60. **Munson, G., and J. Scott.** 1999. Binding site recognition by Rns, a virulence regulator in the AraC family. J. Bacteriol. **181:**2110–2117.
61. **Oh, M. H., S. M. Lee, D. H. Lee, and S. H. Choi.** 2009. Regulation of the *Vibrio vulnificus hupA* gene by temperature alteration and cyclic AMP receptor protein and evaluation of its role in virulence. Infect. Immun. **77:**1208–1215.
62. **O'Neill, M. C.** 1998. A general procedure for locating and analyzing protein-binding sequence motifs in nucleic acids. Proc. Natl. Acad. Sci. U. S. A. **95:**10710–10715.
63. **Puente, J. L., D. Bieber, S. W. Ramer, W. Murray, and G. K. Schoolnik.** 1996. The bundle-forming pili of enteropathogenic *Escherichia coli*: transcriptional regulation by environmental signals. Mol. Microbiol. **20:**87–100.
64. **Russell, R., F. Sharp, and D. Rasko.** 2007. QseA and GrlR/GrlA regulation of the locus of enterocyte effacement genes in enterohemorrhagic *Escherichia coli*. J. Bacteriol. **189:**5387–5392.
65. **Saitoh, T., et al.** 2008. Transcription of the *ehx* enterohemolysin gene is positively regulated by GrlA, a global regulator encoded within the locus of enterocyte effacement in enterohemorrhagic *Escherichia coli*. J. Bacteriol. **190:**4822–4830.
66. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U. S. A. **74:**5463–5467.
67. **Schnaitman, C. A.** 1970. Protein composition of the cell wall and cytoplasmic membrane of *Escherichia coli*. J. Bacteriol. **104:**890–901.
68. **Schultz, S. C., G. C. Shields, and T. A. Steitz.** 1991. Crystal structure of a CAP-DNA complex: the DNA is bent by 90 degrees. Science **253:**1001–1007.
69. **Sherlock, O., R. Vejborg, and P. Klemm.** 2005. The TibA adhesin/invasin from enterotoxigenic *Escherichia coli* is self recognizing and induces bacterial aggregation and biofilm formation. Infect. Immun. **73:**1954–1963.
70. **Skorupski, K., and R. K. Taylor.** 1997. Cyclic AMP and its receptor protein negatively regulate the coordinate expression of cholera toxin and toxin-coregulated pilus in *Vibrio cholerae*. Proc. Natl. Acad. Sci. U. S. A. **94:**265–270.
71. **Stewart, G. S., S. Lubinsky-Mink, C. G. Jackson, A. Cassel, and J. Kuhn.** 1986. pHG165: a pBR322 copy number derivative of pUC8 for cloning and expression. Plasmid **15:**172–181.
72. **Suck, D.** 1997. DNA recognition by structure-selective nucleases. Biopolymers **44:**405–421.
73. **White, C. E., and S. C. Winans.** 2007. The quorum-sensing transcription factor TraR decodes its DNA binding site by direct contacts with DNA bases and by detection of DNA flexibility. Mol. Microbiol. **64:**245–256.
74. **Wickstrum, J., and S. Egan.** 2002. Ni^{+}-affinity purification of untagged cAMP receptor protein. Biotechniques **33:**728–730.
75. **Zhan, L., et al.** 2008. The cyclic AMP receptor protein, CRP, is required for both virulence and expression of the minimal CRP regulon in *Yersinia pestis* biovar microtus. Infect. Immun. **76:**5028–5037.
76. **Zheng, D., C. Constantinidou, and J. Hobman.** 2004. Identification of the CRP regulon using in vitro and in vivo transcriptional profiling. Nucleic Acids Res. **32:**5874–5893.